PCT/IL2004/000642

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APPLICATION NUMBER: ***60/487,956***
FILING DATE: ***July 18, 2003***

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FEE RECORD SHEET

07/21/2003 KBETEMA1 00000077 501407 60487956
01 FC:2005 80.00 DA

PTO-1556
(5/87)

*U.S. Government Printing Office: 2002 — 489-267/69033

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a **PROVISIONAL APPLICATION FOR PATENT** under 37 CFR 1.53 (b)(2).

Docket Number	26421	Type a plus sign (+) inside this box ->	+

INVENTOR(s) / APPLICANT(s)

LAST NAME	FIRST NAME	MIDDLE INITIAL	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
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TITLE OF THE INVENTION (280 characters max)

A DERMASEPTIN DERIVATIVE (DD_{13}) PREVENTS GRAFT ASSOCIATED INFECTIONS OF ANTIBIOTIC-RESISTANT STAPHYLOCOCCI

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ENCLOSED APPLICATION PARTS *(check all that apply)*

☑ Specification	*Number of Pages*	23	☑ Applicant is entitled to Small Entity Status	
☑ Drawing(s)	*Number of Sheets*	4	☑ Other (specify)	3 Claims

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT *(check one)*

☐ A check or money order is enclosed to cover the filing fees		FILING FEE AMOUNT ($)	$ 80.-
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The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.

☑ No

☐ Yes, the name of the US Government agency and the Government contract number are: _____

Respectfully submitted,

SIGNATURE _____ 16 July 2003

25,457
REGISTRATION NO.
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TYPED or PRINTED NAME SOL SHEINBEIN

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INTRODUCTION

Documentation of nosocomial infections due to *Staphylococcus aureus* and *Staphylococcus epidermidis* is increasing over the last decades (39, 34). These common skin inhabitant bacteria are known to be a frequent cause of infections related to prosthetic and indwelling medical devices (14, 43). Several infections are related to bacterial biofilm formation on material surfaces, such as those of venous or urinary catheters, prosthetic heart valves, orthopaedic devices, and even contact lenses (14, 43, 61, 38). In particular, the late-appearing vascular graft infections are one of the most feared complications that the vascular surgeon treats, frequently resulting in prolonged hospitalization, organ failure, amputation, and death (7, 32). Effective strategies for the prevention of prosthetic infection vary from device to device, even though, the capacity of staphylococci to produce a biofilm often makes them resistant to conventional antimicrobial agents (66, 41). The centerpiece of therapy is prophylactic systemic antibiotics. However, recent prophylactic strategies suggested the use of antimicrobials bound in high concentrations to prosthetic grafts have been proposed as added prophylaxis (60, 11, 68). Several studies have focused on developing new prosthetic materials that reduce adhesion or survival of bacteria (68, 26). One of the most interesting studies was based on the fact that resistance of biofilm to antimicrobial agents is acquired as a multi-cellular strategy that relies on exchange of chemical signals between cells in a process known as quorum sensing (45). Thus, interfering with this mechanism of bacterial cell-cell communication could provide a novel approach to prevent biofilm formation. Recently, a seven amino acid peptide termed RNAIII inhibiting peptide (RIP) was described to affect diseases caused by *S. aureus* and *S. epidermidis* (5, 2, 27, 70, 3, 24, 6).

RIP was shown to inhibit *S. aureus* and *S. epidermidis* pathogenesis (biofilm formation (6) and toxin production (70)) by disrupting quorum sensing mechanisms through inhibition of TRAP phosphorylation (4). TRAP is a protein unique to staphylococci but is

highly conserved among strains and species, and therefore its inhibitor RIP was effective against any staphylococcal strain so far tested (2, 27, 70, 3, 24). RIP does not directly kill the bacteria but interferes with its signal transduction, thus making it non pathogenic.

The ubiquitously produced in nature cationic antimicrobial peptides (9, 54, 28, 29, 21, 1, 37, 69) are attracting increasing research and clinical interest for their roles in innate immunity and for their potential uses in various antimicrobial fields (47, 71). The cytoplasmic membrane was proposed to be their ultimate target. Being cationic, the peptides are able to interact electrostatically with the negatively charged phospholipid headgroups and then insert into the membrane bilayer in a manner that leads to its disruption (40, 31, 33, 62). Although the steps involved in this mechanism remain to be delineated, there is a large body of experimental data demonstrating antimicrobial peptides to display a direct correlation between antibiotic effect and increased plasma membrane permeability, conductance of ions across lipid bilayers and dissipation the trans-membrane electric potential (59, 37, 46, 55, 20, 65, 56, 63, 37, 22, 16). Thus, while their precise mechanism of action is not fully understood, their microbicidal effect is believed to result from the peptides capacity to disrupt the ordered membrane structure of target cells. The molecular basis for selective activity between mammalian and bacterial cells is also ill defined, but is believed to result from differences in the lipid composition of target versus non-target cells, such as membrane fluidity and high negative charge density (1, 71, 42, 12). These differences seem to be responsible for the preferred accumulation of antimicrobial peptides on microbial membranes. Numerous studies have demonstrated that the peptides physicochemical properties, i.e., amphipathy, positive charge content and hydrophobicity are the main factors affecting membrane-lysis activity (1, 42, 12, 8). Accordingly, isomers composed of all D-amino acids are as active as the L-enantiomers, implying that the mechanism of action is not mediated by interaction with a

stereo-specific receptor. These properties enable cationic antimicrobial peptides to escape microbial mechanisms involved in multidrug resistance (12, 23, 53).

Dermaseptins are a large family of linear polycationic peptides from frog skin (50, 51, 48, 10) whose cytolytic activity is believed to result from interaction of their N-terminal domain with the plasma membranes of target cells (49, 52). Recent investigations with respect to the relations between physical properties (structure and organization in solution) of dermaseptin S4 and its interaction with target membranes (18, 35) led to the design of short derivatives that maintain the amphipathic alpha-helical structure of the parent peptide, bind avidly to model membranes with association affinity constants (K_A) in the range of 10^5 to 10^7 M^{-1} and exert rapid cytolytic activity against a variety of pathogens. Among these, the 13-mer derivative K_4-S4(1-13)$_a$ is of particular interest as it is the smallest derivative that combines low toxicity and efficient large-spectrum antimicrobial activity both in culture and in animal models of infection (53, 19).

In this disclosure, I show that RIP and the 13-mer dermaseptin derivative DD_{13} individually inhibit biofilm formation on a Dacron graft and that the bactericial peptide efficiently eradicates drug resistant staphylococcal infections.

MATERIALS and METHODS

Peptides: Peptides were synthesized by the solid phase method as described (35), applying the Fmoc active ester chemistry on a fully automated, programmable "Model 433A Peptide Synthesizer" (Applied Biosystems). MBHA-resin (Novabiochem, Germany) was used to obtain amidated peptides. The crude peptides were extracted from the resin with 30% acetonitrile in water and purified to chromatographic homogeneity in the range of 98 to >99% by reverse-phase HPLC (Alliance-Waters). HPLC runs were performed on a semipreparative C4 column (Vydac) using a linear gradient of acetonitrile in water (1%/min), both solvents

containing 0.1% trifluoroacetic acid. The purified peptides were subjected to amino acid analysis and electrospray mass spectrometry in order to confirm their composition. Peptides were stored as a lyophilized powder at -20°C.

Bacteria: Methicillin-resistant *S. aureus* (MRSE) ATCC 43300 was commercially purchased from Oxoid S.p.A., Milan, Italy. Methicillin-resistant *S. epidermidis* (MRSE) is a clinical strain from the Institute of Infectious Diseases and Public Health, University of Ancona, Italy.

Animals: Adult male Wistar rats, 250-300gr (I.N.R.C.A. I.R.R.C.S. animal facility, Ancona) were used, with 15 animals per experimental group.

Susceptibility testing: The antimicrobial susceptibilities of the strains were determined by using the microbroth dilution method, according to the procedures outlined by the National Committee for Clinical Laboratory Standards. The minimal inhibitory concentration (MIC) was taken as the lowest antibiotic concentration at which observable growth was inhibited. Experiments were performed in triplicate.

Graft infection rat model: Rats were anesthetized and their hair of the back was shaved and the skin cleansed with 10% povidone-iodine solution. One subcutaneous pocket was made on each side of the median line by a 1.5 cm incision. Aseptically, 1-cm^2 sterile albumin-sealed Dacron grafts (Albograft ™, Sorin Biomedica Cardio, S.p.A., Saluggia VC, Italy) were implanted into the pockets. Before implantation grafts were soaked for 20 minutes in sterile solutions of the various agents. The pockets were closed by means of skin clips and saline solution (1 mL) containing MRSA or MRSE at a concentration of 2×10^7 cfu/mL was inoculated onto the graft surface by using a tuberculin syringe to create a subcutaneous fluid-filled pocket. The animals were returned to individual cages and thoroughly examined daily. All grafts were explanted at 7 days following implantation.

Assessment of the infection: The explanted grafts were placed in sterile tubes, washed in sterile saline solution, placed in tubes containing 10 mL of phosphate-buffered saline solution and sonicated for 5 minutes to remove the adherent bacteria from the grafts (6). Quantitation of viable bacteria was performed by culturing serial dilutions (0.1 mL) of the bacterial suspension on blood agar plates. All plates were incubated at 37°C for 48 hours and evaluated for the presence of the staphylococcal strains. The organisms were quantitated by counting the number of colony-forming units (CFUs) per plate. The limit of detection for this method was approximately 10 CFU/mL.

Peptides binding to Dacron: To determine how much peptide impregnates the graft, 1 cm^2 slices of collagen-sealed Dacron (AlbograftTM, Sorin Biomedica Cardio, S.p.A., Saluggi VC, Italy) were soaked at room temperature in peptide solutions, in quadruplicates, for 0.5 or 5 hrs (1 mL saline per graft, containing 50 μg/mL of each peptide). Following the incubation period the Dacron was removed and residual peptide (unbound fraction) was subjected to reversed phase-HPLC analysis as described above. Peptide identification was based on retention time and spectral analysis. The amount of unbound peptide was calculated by area integration of the UV absorbing peak (220 nm) and comparison with standard curves of known concentrations for each peptide (18).

RNAIII synthesis and bacterial growth: *S. aureus* cells containing the *rnaiii::blaZ* fusion construct (27) (30μl, $2x10^7$ cells) were grown for 2.5 hrs with 5μL peptides or control buffer. A sample (5μL) was removed, diluted in saline, and streaked on LB agar plates to determine CFU. The rest of the reaction mixture was used to determine RNAIII synthesis (beta-lactamase activity). This was done by adding a substrate of beta-lactamase (nitrocefin), and OD determined at 490nm/650nm (27).

Statistical analysis: MIC values are presented as the geometric mean of three separate experiments. Quantitative culture results from all groups were presented as mean ± standard

deviation and the statistical comparisons between groups were made using analysis of variance (ANOVA) on the log-transformed data. Significance was accepted when the *P* value was ≤0.05.

RESULTS

Prevention of graft-associated infections

The peptides DD_{13} and RIP (Table 1) were tested for efficacy in preventing staphylococcal graft-associated infections. Collagen-coated Dacron grafts were coated with 10, 20 or 50 mg/L of either RIP or DD_{13} and were implanted in rats. Bacteria (MRSA or MRSE) were injected into the implants, implants removed after a week, and bacterial load determined. The study included a negative control group (untreated graft with no bacterial challenge) and a positive control group (untreated graft with bacterial challenge). The results are summarized in Figure 1. None of the animals included in the negative control group had anatomic or microbiological evidence of graft infection (no graft contamination). All rats included in the positive control groups that were implanted with untreated grafts and challenged with MRSA or MRSE (total of 30 rats) demonstrated evidence of graft infection, with quantitative culture results showing $4.4\times10^6 \pm 1.2\times10^6$ and $6.9\times10^6 \pm 1.8\times10^6$ CFU/ml graft, respectively.

Animals implanted with grafts presoaked in 10, 20 or 50 mg/L RIP solutions and challenged with MRSA, demonstrated reduced evidence of infection with $4.1 \times 10^4 \pm 7.1 \times 10^3$, $5.9 \times 10^3 \pm 1.7 \times 10^3$ and $8.4 \times 10^1 \pm 3.6 \times 10^1$ CFU/ml respectively, and those challenged with MRSE demonstrated reduced evidence of infection with $6.9 \times 10^3 \pm 1.9 \times 10^3$, $8.5 \times 10^2 \pm 2.0 \times 10^2$ and $3.9 \times 10^1 \pm 1.6 \times 10^1$ CFU/ml, respectively.

Animals implanted with grafts presoaked with DD_{13} and challenged with MRSA, demonstrated reduced or no evidence of infection with $5.2\times10^2 \pm 1.6\times10^2$, $4.0\times10^1 \pm 1.7\times10^1$ CFU/ml and negative quantitative cultures, respectively for the same doses as RIP.

Similarly, animals challenged with MRSE, demonstrated reduced or no evidence of infection with $5.2\times10^2 \pm 1.6\times10^2$, $4.4\times10^1 \pm 1.3\times10^1$ CFU/ml and negative quantitative cultures, respectively.

In addition, treatment with these peptides was compared with the conventionally used antibiotic, Rifampin (60). These experimental groups received grafts pre-soaked with 5 mg/L rifampin alone, or rifampin and RIP or DD_{13} at 10 mg/L. The group of rifampin-treated rats showed quantitative culture of $6.7\times10^3 \pm 9.1\times10^2$ CFU/mL MRSA and $8.8\times10^2 \pm 3.0\times10^2$ CFU/mL MRSE, but when soaked in combination with RIP ot DD_{13}, animals showed negative quantitative cultures and no evidence of infection.

Of note is that all agents used did not show any signs of toxicity and none of the animals included in any group died or had clinical evidence of drug related adverse effects, such as local signs of perigraft inflammation, anorexia, vomiting, diarrhea, and behavioral alterations. Reduction in bacterial load was significant ($p<0.05$) in all experimental groups when compared to positive control groups.

In vitro studies

Peptide binding to Dacron cuffs: To verify the relationships between the observed in vivo activity and the amount of peptide present on the Dacron grafts, the grafts were soaked in peptide solutions as described above prior to be grafted in rats. After removing the grafts, the solutions were subjected to HPLC analysis from which the amount of Dacron-bound peptide was deduced from calculated amount of residual unbound fraction. The results are summarized in Figure 3 for a peptide solution of 50 mg/L. RIP bound the least with a mean bound amount of 6.5 ± 2.5 $\mu g/cm^2$. DD_{13} was found to bind more significantly than RIP with a

mean bound amount of 27±0.5 μg/cm^2. The data indicates that longer soaking time periods enable uptake of larger amounts of each peptide. For instance, nearly 100% (i.e., 50 μg) of DD_{13} was found to bind after 5 hours soaking (data not shown). While these experiments indicate that in the future, longer soaking time will result in higher peptide concentrations on the grafts, they also demonstrate that the higher protective efficacy of the bactericidal peptide as compared to RIP is not due to its relative concentration but rather to its specific activity (see below).

Bacterial growth and RNAIII synthesis: To better understand the molecular mechanisms involved in the observed activity, the peptides were investigated for their effect(s) in vitro on RNAIII synthesis (known to be inhibited by RIP) and for bacterial proliferation (known to be inhibited by DD_{13}). These experiments were performed by growing *S. aureus* cells containing an *rnaiii::blaZ* fusion construct in the presence of either one the peptides. Cultures were followed by determination of RNAIII synthesis by a colorimetric method using nitrocefin as a substrate while the peptides effect on bacterial viability was assessed by performing CFU counts using conventional microbiological methods. The results are summarized in Figure 4.

DD_{13} displayed potent bactericidal activity whereas RIP was virtually inactive (Fig. 4, panel A). According to the broth-microdilution method, DD_{13} exhibited a MIC at 2 mg/L for both staphylococcal strains (as compared to susceptibility to rifampin of MIC values of 0.5 mg/L for both of the organisms). RIP did not demonstrate any in vitro bactericidal activity against neither of the two strains, when tested up to 128 mg/L (data not shown).

As also shown in Fig. 4, RIP efficiently inhibited RNAIII synthesis while DD_{13} appeared to be more efficient than RIP. However, closer inspection of the data revealed that at high peptide concentrations, most of the inhibitory activity observed was attributable to cell

death. Moreover, at low peptide concentrations, where no cell death occurred, DD_{13} was unable to affect RNAIII synthesis (Inset of Figure 4, panel B).

DISCUSSION

In order to prove efficacy in preventing bacterial adhesion and biofilm formation in vivo, a well characterized experimental Dacron graft rat model was used. For comparison purposes the antibiotic rifampin was chosen for its current utilization in clinical practice against *staphylococci* (60). Rifampin was very effective in our experimental model, as expected from previous literature (3, 24). However, like with RIP alone, rifampin did not eradicate bacterial infection. 100% inhibition was reached only when rifampin was combined with a peptide. The reason why RIP did not cause total inhibition when used alone could be due to the actual amount of RIP bound to the graft. It is estimated that soaking the graft in 50 mg/L RIP resulted in 6.5 ± 2.5 μg RIP bound to the graft before implantation, which may not be enough to prevent adhesion of the 10 million cells that were injected. Perhaps if the grafts were coated with a higher concentration of RIP such as after longer soaking time, better prevention could have been reached with RIP and other peptides. Note however, that such a high level of a single dose of bacterial contamination is unlikely to occur in clinical settings, suggesting that the amounts of bound peptides as utilized in the present in vivo experiments could be sufficient to pre-coat grafts in clinical practice..

The present study also demonstrates that like RIP, the 13-residue dermaseptin derivative DD_{13} is highly efficient in inhibiting staphylococcal infections whether used alone or in combination with the conventional antibiotic. In fact, DD_{13} was significantly more efficient than RIP. According to our estimation, the amount of peptide present on the graft excludes the possibility that efficacy was proportional to some higher quantity of bound peptide. Thus, while DD_{13} had about threefold higher binding than RIP, efficacy in-vivo was

at least tenfold higher at each one of the concentrations used. This suggests that the high efficacy of DD_{13} in-vivo is not due to its relative concentration on the graft but rather to its specific activity.

In addition, the use of DD_{13} might be preferable over RIP due to the peptide's ability to kill bacteria and simultaneously neutralize the threat that the bacteria might release endotoxins which can cause septic shock upon their introduction into the bloodstream. Antimicrobial peptides such the dermaseptin are known for their ability to bind endotoxins and neutralize them and thereby prevent septic shock.

In conclusion, I show in this disclosure an alternative approach to preventing device-associated infection by *S. aureus* and *S. epidermidis*. Since DD_{13} is highly effective in preventing staphylococcal infections, our data suggests that DD_{13} could be used to coat medical devices known to be associated with staphylococcal infections.

FOOTNOTES

Acknowledgments: This research was partly supported by the DA'AT consortium, a Magnet project administrated by the Office of the Chief Scientist at the Ministry of Industry, and partly by the Technion's President Research Fund.

Key Words : Antimicrobial peptides; Bacteria; Quorum sensing; RNAIII inhibiting peptide; Dacron; Biofilm.

Abbreviations: RIP: RNAIII inhibiting peptide; DD_{13}: a 13-mer dermaseptin derivative; MRSA: methicillin-resistant *Staphylococcus aureus;* MRSE: methicillin-resistant *S. epidermidis*; TRAP: target of RNAIII-activating protein; Fmoc: 9-fluorenylmethyloxycarbonyl; MBHA: 4-methylbenzhydrylamine. CFU : colony forming unit. CD: circular dichroism.

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FIGURE LEGENDS

Figure 1. Peptides effect on graft associated infections in rats. Dacron grafts were pre-soaked in saline (control), RIP or DD_{13}, at the designated concentrations and implanted in rats. Grafts were then inoculated with methicillin-resistant *S. aureus* (MRSA) or a clinical isolate of methicillin-resistant *S. epidermidis* (MRSE). Grafts were removed after a week and assessed for bacterial load. Plots show typical counts of viable CFUs for MRSA (triangles) or MRSE (circles). Statistical data is detailed in Results section.

Figure 2. Prevention of graft associated infections in rats based on combined therapy. Grafts pre-soaked in saline (control), rifampin alone (5 mg/L) or rifampin combined with either RIP or DD_{13} (10 mg/L) were implanted in rats and inoculated with MRSA or MRSE. Grafts were removed after a week and assessed for bacterial load. Plots show the resulting counts of viable CFU. Statistical data is detailed in Results section. A star indicates negative quantitative cultures.

Figure 3. Peptides binding to Dacron: Collagen-sealed Dacron grafts were soaked in peptide solutions (50 mg/L). Bound peptide was estimated from unbound fraction that was subjected to analytical reversed-phase HPLC. Peptide identification was based on retention time and spectral analysis. Unbound peptide was determined after area integration of the UV absorbing peak (220 nm) and comparison with standard curves of known concentrations (18). Error bars indicate standard deviations of the mean determined from four independent experiments.

Figure 4. In-vitro effects of peptides on bacterial growth and RNAIII synthesis. *S. aureus* cells containing *rnaiii::blaZ* fusion construct (~$2x10^7$ cells) were grown with peptides or control buffer then divided to two portions. One portion was diluted in saline and streaked on LB agar plates to determine CFU (panel A). The other portion was used to determine RNAIII synthesis (panel B) by adding the beta-lactamase substrate nitrocefin, and measuring

OD at 490nm/650nm (27). Error bars indicate standard deviations from the mean as determined from two independent experiments performed in triplicates. Inset: Same as panel B focussing on low peptide concentrations. Symbols in panel B are as in panel A.

Claims:

1. The use of a dermaseptin derivative essentially as described herein and its functional analogs or equivalents for preventing graft associated infections of antibiotic-resistant *staphylococci.*
2. The use of a dermaseptin derivative essentially as described herein and its functional analogs or equivalents for treating bacterial infections.
3. The use of a dermaseptin derivative essentially as described herein and its functional analogs or equivalents for treating *staphylococci* infections.

Table 1

Primary structures and designations of the peptides investigated.

Peptide	Amino acid sequence	Designation
RIP	$YSPWTNF_{CONH2}$	RIP
K_4-S4 (1-13)$_a$	$ALWKTLLKKVLKA_{CONH2}$	DD_{13}

A DERMASEPTIN DERIVATIVE (DD_{13}) PREVENTS GRAFT ASSOCIATED INFECTIONS OF ANTIBIOTIC-RESISTANT *STAPHYLOCOCCI*

Inventor: Amram Mor

ABSTRACT

Staphylococcal bacteria are a prevalent cause of infections associated with foreign bodies and indwelling medical devices. Bacteria are capable of escaping antibiotic treatment through encapsulation into biofilms. RIP is a heptapeptide that inhibits staphylococcal biofilm formation by obstructing quorum sensing mechanisms. K_4-S4(1-13)$_a$ is a 13-residue dermaseptin derivative (DD_{13}) believed to kill bacteria via membrane disruption. I tested each of these peptides for their ability to inhibit bacterial proliferation and suppress quorum sensing in vitro, and for their efficacy in preventing staphylococcal infection in a rat graft infection model using *S. aureus* (MRSA) or *S. epidermidis* (MRSE). In vitro, proliferation assays demonstrated that RIP had no inhibitory effect while, at least at non-cytolytic doses, DD_{13} was unable of inhibiting RNAIII synthesis, a regulatory RNA molecule important for staphylococcal pathogenesis. In vivo, both peptides reduced graft associated bacterial load in a dose dependant manner but the bactericidal peptide was more potent in totally preventing *Staphylococcal* infections at the lowest dose. In addition, both peptides acted synergistically with antibiotics. The data indicates that DD_{13} may be useful for coating medical devices to prevent drug-resistant staphylococcal infections.

Figure 1.

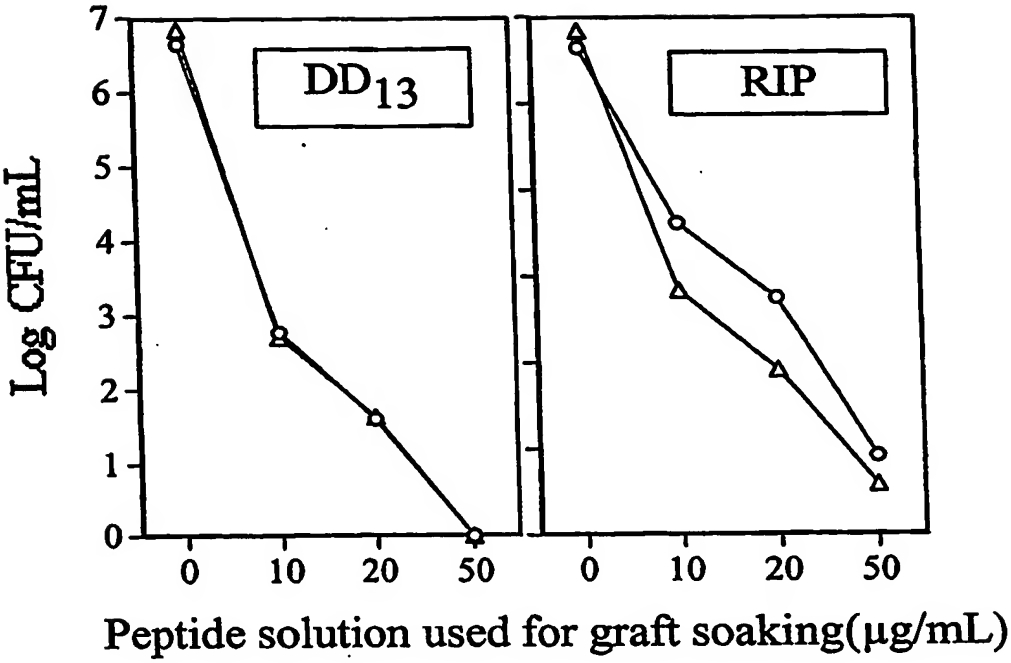

DD13
RIP
Log CFU/mL
0
1
2
3
4
5
6
7
0
10
20
50
0
10
20
50
Peptide solution used for graft soaking(µg/mL)

Figure 2

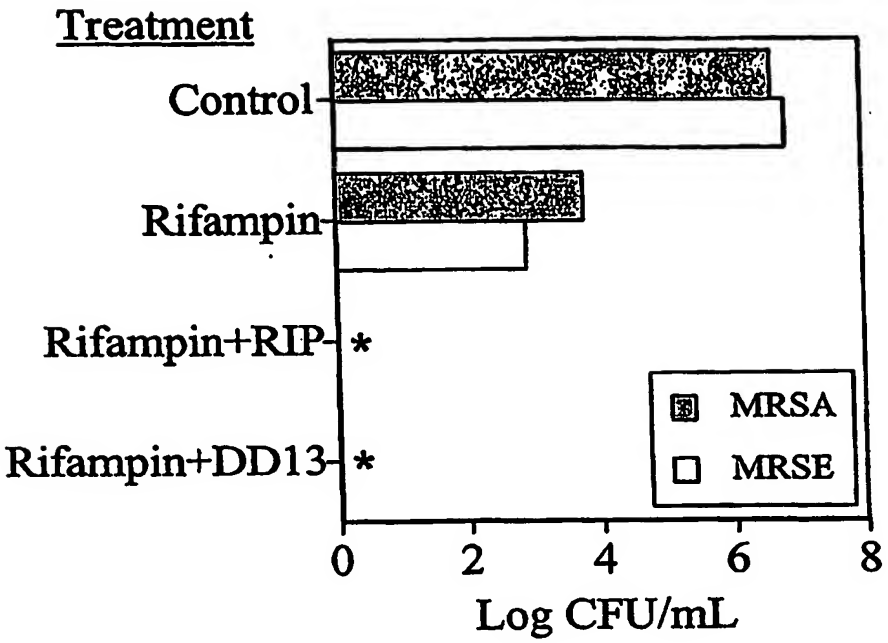

Treatment
Control
Rifampin
Rifampin+RIP
Rifampin+DD13
*
*
MRSA
MRSE
0
2
4
6
8
Log CFU/mL

Figure 3

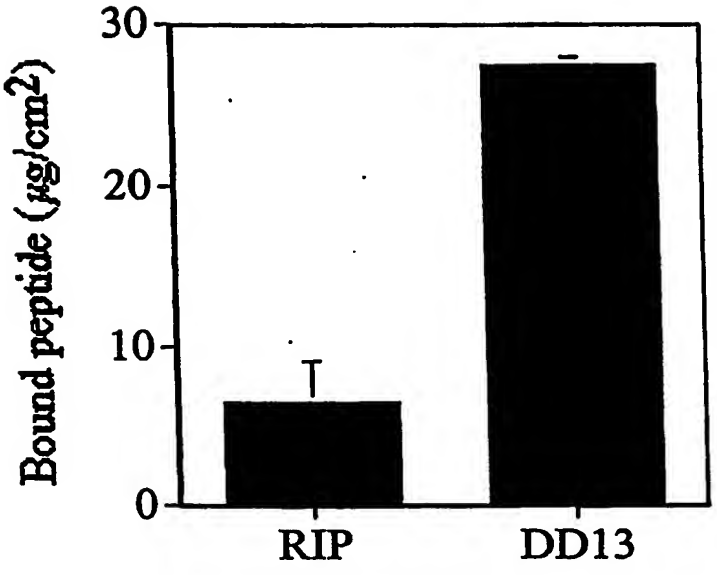
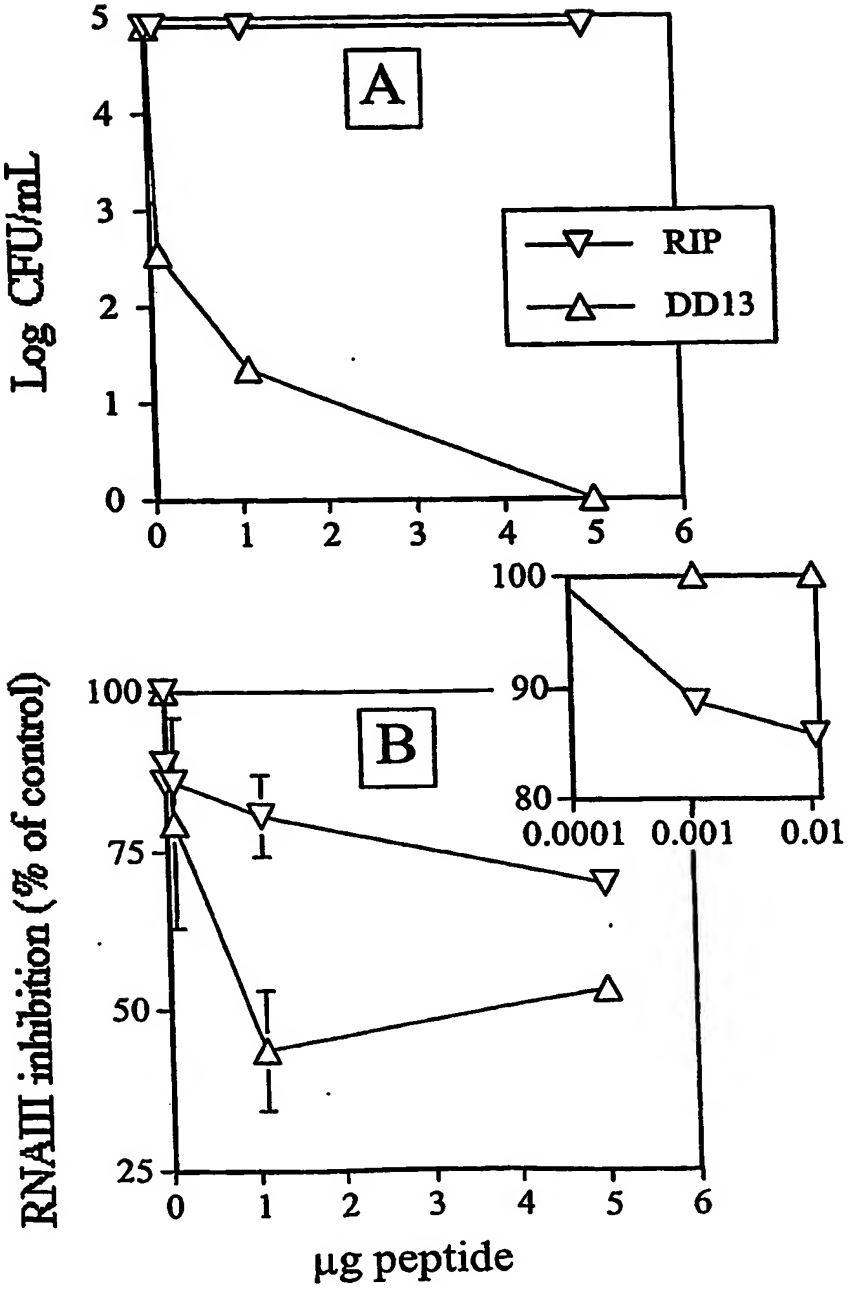


Figure 4


A
B
Log CFU/mL
RIP
DD13
RNAIII inhibition (% of control)
µg peptide
0
1
2
3
4
5
6
25
50
75
80
90
100
0.0001
0.001
0.01